INVASION OF EPITHELIAL CELLS BY *TRICHINELLA SPIRALIS*: *IN VITRO* OBSERVATIONS

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Summary :

It has been known for many years that *Trichinella spiralis* initiates infection by penetrating the columnar epithelium of the small intestine, however, the mechanisms used by the parasite in the establishment of its intramulticellular niche in the intestine are unknown. The recent demonstration that invasion also occurs *in vitro* when infective larvae of *T. spiralis* are inoculated onto cultures of epithelial cells provides a model that allows the direct observation of the process by which the parasite recognizes, invades and migrates within the epithelium. The finding that penetration of the cell membrane or induction of plasma membrane wounds by larvae do not always result in invasion argue in favor of some kind of host-parasite communication in successful invasion. In this sense, the *in vitro* model of invasion provides a readily manipulated and controlled system to investigate both parasite, and host cell requirements for invasion.

KEY WORDS : *Trichinella spiralis*, invasion, epithelial cells, *in vitro*.

The intestinal epithelium is the first site of host contact for the parasitic nematode *Trichinella spiralis*. Invasion of epithelial cells by infective L1 larvae is crucial for the establishment of infection. Studies of nematode establishment of intestinal niches has been hindered by the lack of a readily manipulated *in vitro* assay. ManWarren *et al.* (1997) described for the first time an *in vitro* model that supports the development of the parasite and reproduces several *in vivo* parameters of the worm establishment. This system allows the direct observation of parasite behavior, provides a method for study of parasite interaction with the host cell, and presents novel opportunities for investigation of host immune responses.

REQUIREMENTS FOR INVASION *IN VITRO*

This system employs monolayers of epithelial cells grown to confluence in glass chamber slides, on coverglasses, or on filters. Infectious larvae, recovered by pepsin digestion and activated by exposure to the intestinal milieu, are suspended in a semisolid medium (1.75 % agarose) and overlaid on cell monolayers. When examined by microscopy, larvae are observed to penetrate plasma membranes and move within the cytoplasm of contiguous cells. Theses observations support earlier conclusions from electron microscopic studies of intestinal tissue that *Trichinella* is an intramulticellular pathogen (Wright, 1979). The presence of the agar is critical for invasion, perhaps by providing mechanical support for the larva during the initial penetration. Without agar, infective larvae move over the epithelial monolayers but do not penetrate the cells (ManWarren *et al.*, 1997; Li *et al.*, 1998). Another requirement for invasion is the activation of larvae by exposure to the small intestine or its contents. Activation can be achieved by inoculating larvae into rats and recovering them from the intestines after a short period of time, or by incubation of larvae with intestinal contents or bile *in vitro* (ManWarren *et al.*, 1997).

LARVAL BEHAVIOR DURING INVASION

Once on the monolayer, an activated larva moves in a serpentine manner and "browses" the cell surface by probing and poking with its head. Recently, experiments using fluorescent dextrans (Fdx) as fluid phase markers showed that the larvae do more than examine the cell surface (Butcher *et al.*, 2000). Cells inoculated with larvae in the presence of Fdx took up significantly more fluorescent marker than cells from uninoculated monolayers. The fact that low molecular weight Fdx (10 kDa) enter the nuclei of such cells indicates that the markers were free in the cytoplasm, and implies a non-vesicular introduction. Larvae may create transient, non-lethal breaches in the plasma membrane, providing a mechanism for molecules to enter the cytoplasm directly from the extracellular milieu.

Larvae exhibit "browsing" behavior on all cell lines we have tested, however, larvae do not always invade the cells they browse. Results of early experiments with

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different cell types (ManWarren *et al.*, 1997) indicated that larvae invaded only epithelial cell lines, however, one rat epithelial cell line, IEC-6, is resistant to invasion. L1 activated larvae actively browse the monolayer but do not enter the cells (Butcher *et al.*, 2000). Comparison of epithelial cell lines revealed different levels of susceptibility to invasion as well as differences in larval behavior in the monolayer (McVay *et al.*, 2000). Madin Darby Canine Kindney epithelial cells (MDCK) are relatively resistant. Larvae invade MDCK soon after inoculation and emerge after traveling a limited distance in the monolayer, leaving short, serpentine trails of dead cells. Henle 407 cells (human, small intestine) appear to be of intermediate susceptibility to invasion. Migrating larvae create serpentine trails of dead cells but are easily separated from the monolayers during the process of fixation and staining. The Caco-2 cell line (human, large intestine) is the most susceptible to invasion. Larvae invade the cells within seconds of inoculation, creating long, contiguous trails, which reflects a prolonged occupation of the intracellular niche. Furthermore, larvae are retained in Caco-2 monolayers during washing and fixation, suggesting that they are sequestered in these cells.

PARASITE PRODUCTS MEDIATING INVASION

In spite of these direct observations of larval behavior, the mechanism of invasion is still unknown. The infective L1 larva lacks oral appendages or a stylet that could facilitate entry into the cell. Furthermore, as described above, plasma membrane wounds induced by the worm's body or head are not sufficient to allow the larva into IEC-6 cells. Thus, it would appear that invasion is not strictly a mechanical process, but that some kind of parasite-host cell communication is required. The participants in this interaction, from the host or the parasite, are not known. It is possible that excretory-secretory products (ESP) released by the larvae during the early stages of the intestinal phase (Despommier, 1983), or larval surface components which are in intimate contact with the cells, facilitate entry into and transit through the epithelium. Many of the proteins in ESP and on the surface bear complex glycans that are capped with an unusual sugar called tyvelose. *In vivo* studies have shown that antibodies able to bind tyvelose protect epithelia from invasion and cause established *T. spiralis* larvae to abandon their niche (Appleton *et al.*, 1988). ManWarren *et al.* (1997) investigated the release of ESP by the L1 larvae during infection of epithelial monolayers. Infected, susceptible monolayers stained with fluorescent-labeled, anti-tyvelose monoclonal antibodies showed heavy glycan deposition in the trails of dead cells left by the larva. When the L1 larvae were co-cultured with anti-tyvelose antibodies, different inhibitory activities were observed: exclusion of larvae from epithelial cells, encumbrance of larvae as they migrate within epithelial monolayers and interference with molting/ecdysis (McVay *et al.*, 2000). These effects correlate with antibody-mediated effects that have been described in passively immunized suckling rats (Carlisle *et al.*, 1991). Excluded larvae bear cephalic caps of immune complexes formed by disgorged glycoproteins and tyvelose-specific antibodies (McVay *et al.*, 1998). These caps may block amphid-mediated sensory reception or act as physical barriers to cell penetration. In either case, the inhibitory effect is an indirect result of immune complex formation by antibody binding to tyvelose. In subsequent experiments, it was shown that exclusion also occurs when monovalent Fab fragments were used and there were no caps evident. This observation implicates surface glycoproteins or ESP as direct mediators in the initiation of the infectious process. In other studies, antibody binding that was limited to surface glycoproteins was not inhibitory, supporting the conclusion that surface structures are not pivotal in cell invasion (McVay *et al.*, 1998). ESP of *T. spiralis* are known to contain several kinds of proteinases (Criado-Fornelio *et al.*, 1992), and an endonuclease activity has been recently described (Mak & Ko, 1999). Although neither of these activities is likely to mediate invasion, it is possible that they may facilitate the worm transit through the epithelium. It is possible that *Trichinella* secretes other proteins that specifically facilitate invasion. For example, a pore-forming protein has been reported to be secreted by the parasitic nematode, *Trichuris* (Drake *et al.*, 1994). This is of particular interest, because the adult stage of *Trichuris* resides in an epithelial site similar to that of *Trichinella* (Lee & Wright, 1978). The presence of a similar protein in *Trichinella* ESP is likely, but has to be described.

CELLULAR REQUIREMENTS FOR INVASION

As described above, ESP seems to play a important role in the invasion process but delivery of ESP to cells is not sufficient to allow the entry of *T. spiralis* into all cells. Butcher *et al.* (2000), showed that activated L1 larvae browsed the surface of resistant IEC-6 monolayers, deposited ESP in the cells but did not penetrate them. This suggests that an appropriate cellular response is required for the invasion. As described above, the larval activity on the apical surface of the IEC-6 monolayer results in transient and non-lethal breaches in plasma membranes. Resealing membrane wounds requires the recruitment of exocytic vesicles to the site of injury (Miyake & McNeil, 1995).

It has been suggested that the larva-induced injury to epithelial cells may induce release of cell-specific mediators that signal the larva to invade a particular cell line or to ignore it (Butcher *et al.*, 2000).

Another possibility is that the binding of ESP to appropriate cellular receptors may be necessary for the initiation of signaling cascades that permit or promote invasion. More information about the nature of the ESP is needed to resolve this question. Although a few genes encoding ESP polypeptides have been cloned (Zarlenga & Gamble, 1990; Arasu *et al.*, 1991; Su *et al.*, 1991; Vassilatis *et al.*, 1992), none of the deduced amino acid sequences show significant homology to any gene of known function. Cloning of individual ESP and surface glycoprotein cDNAs, and the analysis of deduced amino acid sequences seems to be a logical step to address this question.

The utility of this model of intestinal parasitism in the study of immunity has been demonstrated in reports from our laboratory (McVay *et al.*, 1998, 2000) and from the laboratory of D. Wakelin (Li *et al.*, 1998). Further elaboration of the model would include co-culture of epithelia with inflammatory and/or lymphoid cells. In addition, the method should be of considerable value in more detailed investigations of intestinal parasitism by larval and adult stages of *T. spiralis*, as well as other nematode parasites.

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